Genetic Mechanisms Underlying Apimaysin and Maysin Synthesis and Corn Earworm Antibiosis in Maize (*Zea mays* L.)

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ABSTRACT

C-glycosyl flavones in maize silks confer resistance (*i.e.*, antibiosis) to corn earworm (*Helicoverpa zea* [Boddie]) larvae and are distinguished by their B-ring substitutions, with maysin and apimaysin being the di- and monohydroxy B-ring forms, respectively. Herein, we examine the genetic mechanisms underlying the synthesis of maysin and apimaysin and the corresponding effects on corn earworm larval growth. Using an F_2 population, we found a quantitative trait locus (QTL), *rem1*, which accounted for 55.3% of the phenotypic variance for maysin, and a QTL, *pr1*, which explained 64.7% of the phenotypic variance for apimaysin. The maysin QTL did not affect apimaysin synthesis, and the apimaysin QTL did not affect maysin synthesis, suggesting that the synthesis of these closely related compounds occurs independently. The two QTLs, *rem1* and *pr1*, were involved in a significant epistatic interaction for total flavones, suggesting that a ceiling exists governing the total possible amount of C-glycosyl flavone. The maysin and apimaysin QTLs were significant QTLs for corn earworm antibiosis, accounting for 14.1% (*rem1*) and 14.7% (*pr1*) of the phenotypic variation. An additional QTL, represented by *umc85* on the short arm of chromosome *6*, affected antibiosis (R^2 = 15.2%), but did not affect the synthesis of the C-glycosyl flavones.

C-GLYCOSYL flavone synthesis occurs via a branch of the phenylpropanoid/flavonoid pathway (Figure 1; Heller and Forkman 1994). The enzymes involved are believed to be associated with the endoplasmic reticulum (Hrazdina and Wagner 1985; Stafford 1990). After synthesis, flavones are either sequestered in vacuoles or secreted into the cell wall (Stafford 1990). The three major C-glycosyl flavones isolated from maize silk tissue are distinguished by their B-ring substitutions: maysin (5,7,3′,4′-tetrahydroxy), apimaysin (5,7,4′-trihydroxy), and methoxymaysin (5,7,4′-trihydroxy 3′-methoxy; Waiss *et al.* 1979, 1981; Elliger 1980a,b; Figure 1). B-ring substitutions occur either at the 9-carbon stage, as in the case of the lignin precursors (4-coumarate [4-hydroxy], caffeic acid [3,4-dihydroxy], and ferulic acid [3-methoxy 4-hydroxy]; for review see Whetten and Sederoff 1995; Campbell and Sederoff 1996), or at the 15-carbon stage. For flavonoids, the prevailing theory is that B-ring substitutions occur at the 15-carbon stage, and that 4-coumarate is the precursor. The enzyme responsible for the addition of the second hydroxyl to the B-ring is flavonoid 3′-hydroxylase (F3′H; Heller and Forkmann 1994; Holton and Cornish 1995). Larson *et al.* (1986) demonstrated that 3′-hydroxylation of anthocyanins in maize aleurone tissues is dependent on the *pr1* locus. Because of the precedent for sharing of common structural enzymes among the flavonoid pathways [*e.g.*, *bz1* in anthocyanin and flavonol synthesis (Larson and Coe 1977; Furtek *et al.* 1988) and *a1* in anthocyanin and 3-deoxy-anthocyanin synthesis (Reddy *et al.* 1987; Schwarz-Sommer *et al.* 1987)], the *pr1* locus may also be involved in hydroxylation of the B-ring in flavone synthesis (Styles and Ceska 1975). Apimaysin and maysin differ only by the presence or absence of the 3′-hydroxyl group, and their synthesis is presumed to occur via the same pathway. Genetic factors that regulate the synthesis of maysin would be assumed to affect the synthesis of apimaysin.

We are studying flavone synthesis as a model for understanding the genetic mechanisms underlying quantitative trait expression (Byrne *et al.* 1996, 1997, 1998; McMullen *et al.* 1998). Flavone synthesis can be monitored directly by identifying and quantifying flavone compounds using reversed-phase HPLC (Snook *et al.* 1989). The agronomic effects of flavone synthesis can be monitored indirectly through corn earworm larval bioassays (Wiseman 1989). Natural resistance to corn earworm has been attributed to high concentrations of C-glycosyl flavones in silk tissue (Waiss *et al.* 1979, 1981; Elliger *et al.* 1980a; Snook *et al.* 1993, 1994, 1995).

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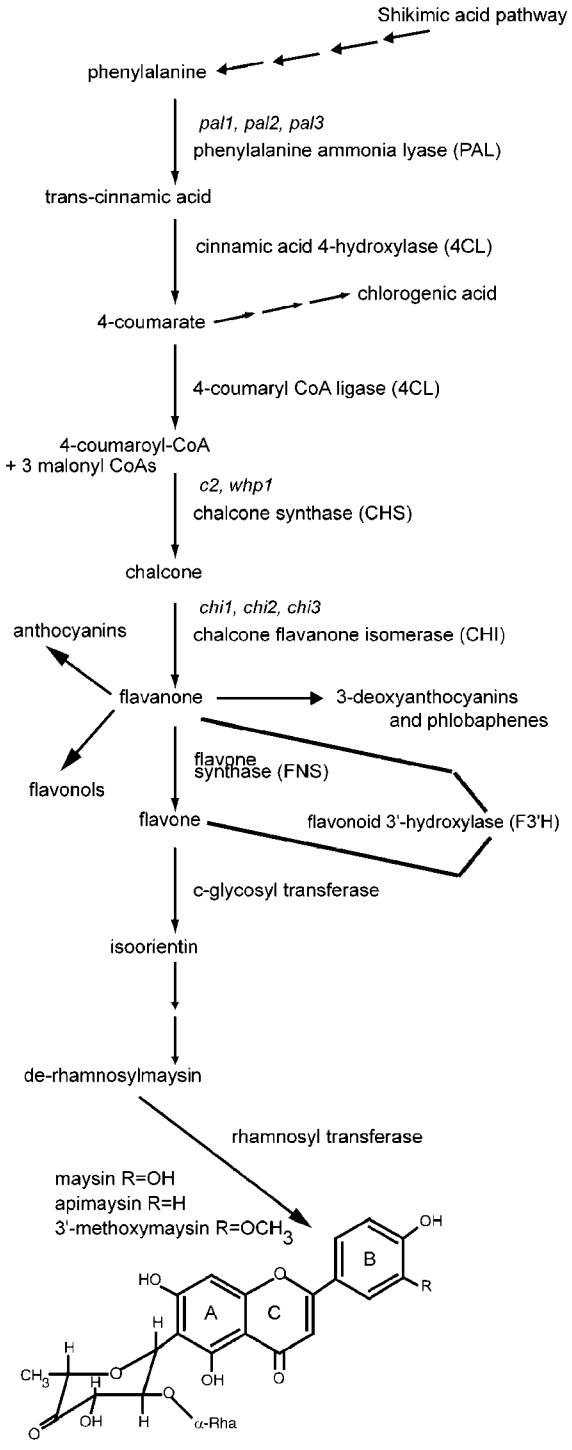

Figure 1.—Flavonoid branch of the phenylpropanoid pathway leading to flavone synthesis: pathway intermediates, enzymatic steps, branch points of competing pathways, known genes, and the basic chemical structure of C-glycosyl flavones are illustrated. Using the anthocyanin/flavonol pathway as a model, flavonoid 3′-hydroxylase is probably acting just upstream or downstream of flavone synthase in the pathway.

C-glycosyl flavones function as antibiotic agents through their subsequent conversion to the more toxic quinones. Quinones reduce the availability of free amino acids and proteins by binding to $-SH$ and $-NH_2$ groups (Felton *et al.* 1989; Wiseman and Carpenter 1995). Antibiosis is characterized by reduced larval and pupal weights and by extended time to pupation (Wiseman and Isenhour 1990). The monohydroxy flavone, apimaysin, is only half as effective in decreasing larval weights as maysin (Elliger *et al.* 1980a,b; Snook *et al.* 1993). Lower efficacy is presumably the result of one fewer hydroxyl group available for oxidation during the conversion to quinones.

Transcriptional activation of the flavone pathway branch is controlled by P1, a myb-like transcription factor encoded by the *p1* locus. There are five allelic variants of *p1* that are distinguished by their tissue specificity patterns (Peterson 1990; Chopra *et al.* 1996; M. D. McMullen, unpublished data). Three of the variants direct flavone synthesis in silk tissue: *P1-rrb* (red pericarp, red cob, browning silks), *P1-wrb* (colorless pericarp, red cob, browning silks), and *P1-wwb* (colorless pericarp, white cob, browning silks; Coe 1985; Coe and Han 1986; M. D. McMullen, unpublished data). The silk browning phenotype observed with these *p1* alleles results from oxidation of accumulated flavones, as often occurs when the tissue is damaged (Levings and Stuber 1971). Byrne *et al.* (1996, 1997) showed that in a population segregating for functional and nonfunctional *p1* alleles, the *p1* locus behaves as a major quantitative trait locus (QTL) for maysin levels and corn earworm larval antibiosis. Fifty-eight percent of the phenotypic variance observed for maysin levels was accounted for by the *p1* locus (Byrne *et al.* 1996), with a high degree of negative correlation between maysin levels and larval weight ($r = -0.92$, $P < 0.0001$; Byrne *et al.* 1997). A second maysin QTL was found on the short arm of chromosome 9, showing dominant gene action for low maysin levels and a significant epistatic interaction with *p1* (Byrne *et al.* 1996). We have named this putative gene *recessive enhancer of maysin1* (*rem1*). (We do not know whether the dominant or recessive *rem1* allele is functional; therefore, both the dominant and recessive alleles are referred to as *rem1*.) In the presence of a functional *P1* allele, recessive *rem1* increased maysin levels nearly twofold. Surprisingly, larval weight was not affected by *rem1*, nor was the *p1* × *rem1* interaction significant for larval weight (Byrne *et al.* 1997). The same effect on maysin and lack of effect on larval weight associated with *rem1* was also observed in a second population (GE37 × FF8), suggesting that the increase in maysin may come at the expense of another unknown antibiotic compound (Byrne *et al.* 1998).

In this paper, we report the results of a QTL study that extends the understanding of flavone synthesis and corn earworm antibiosis, as well as the genetic and cellular mechanisms involved in quantitative trait expression. Specifically, our objectives were to examine the following: (1) the importance of allelic variants in a structural enzyme by testing if *pr1* is involved in flavone synthesis

and if it is a QTL for both apimaysin and maysin synthesis; (2) the nature of epistatic interactions for QTLs for maysin, apimaysin, and total flavone levels; and (3) the consequences of varying flavone forms and levels on corn earworm antibiosis.

MATERIALS AND METHODS

Mapping population: The F_2 population was developed from a cross between the inbred lines GT114 and NC7A. GT114 was developed at the Insect Biology and Population Management Research Laboratory, USDA-ARS, Tifton, GA (Widstrom *et al.* 1988). GT114 has moderately high maysin levels and negligible apimaysin levels in silk tissues (Figure 2). NC7A was derived by N. W. Widstrom from the line NC7 and was developed by the North Carolina Agricultural Research Service, North Carolina State University, Raleigh, NC (Henderson 1976). NC7A has moderately high apimaysin and maysin levels in silk tissues (Figure 2). Testcrosses indicated that GT114 has a functional *Pr1* allele and a *P1-wrb* (colorless pericarp, red cob, browning silks) allele at *p1*, and that NC7A has a nonfunctional *pr1* allele and a *P1-wwb* (colorless pericarp, white cob, browning silks) allele at the *p1* locus. GT114 does not contain the *rem1* allele that increases maysin (Byrne *et al.* 1996). NC7A's constitution at *rem1* was unknown. The 316 (GT114 $\times$ NC7A)F_2 individuals used in this study were derived from a single self-pollinated F_1 plant.

Tissue collection and chemical analysis: The (GT114 $\times$ NC7A)F_2 plants were grown at the University of Missouri Agronomy Research Center near Columbia, Missouri during the summer of 1996. Two replications of GT114, NC7A and (GT114 $\times$ NC7A)F_1 were grown in rows adjacent to the F_2 plot. Leaf tissue was collected from F_2 individuals at the midwhorl stage for RFLP analysis. Emerging primary ear shoots were covered to prevent pollination. Silk tissue was collected 2 days after emergence from the husks. Silk masses were collected into preweighed screw-cap 50-ml tubes, placed on ice for transport to the laboratory, weighed, stored in a $-80°$ freezer, and lyophilized. Lyophilized samples were shipped to the Phytochemical Research Unit, USDA-ARS (Athens, GA) for chemical analysis. The lyophilized silk masses were extracted with 50 ml methanol at 0° for 14 days. Extract concentrations of maysin, apimaysin, and methoxymaysin were determined by reversed-phase HPLC (Snook *et al.* 1989, 1993) and expressed as percent fresh silk weight. After silk collection, F_2 ears were self-pollinated to generate $F_{2:3}$ families.

RFLP analysis: The DNA extraction and Southern hybridization procedures were as described in Byrne *et al.* (1996). Eighty-five DNA probes gave 88 codominant polymorphisms (three duplicate loci). Included in the set of 85 DNA probes were the genomic or cDNA clones for five loci involved in the flavonoid pathway (*r1*, *bz1*, *c2/whp1*, and *p1*). In addition, we used one simple sequence repeat primer pair that gave a codominant polymorphism. Reaction conditions for simple sequence repeat markers were as follows: 50 ng of each primer (Research Genetics, Huntsville, AL), 0.3 units of AmpliTaq Gold polymerase (Perkin Elmer, Norwalk, CT), 1X AmpliTaq Gold buffer, 50 ng genomic DNA, 1.6 mm $MgCl_2$, 0.1 mm of each dNTP, and sterile H_2O to a total volume of 15 μl. Cycling conditions: 10-min dwell at 95°, two cycles of (1 min at 95°, 1 min at 65°, 1.5 min at 72°), 10 cycles with a 1° decrement in the annealing temperature per cycle down to an annealing temperature of 55°, and 30 cycles of (1 min at 95°, 1 min at 55°, 1.5 minutes at 72°). Reactions were carried out in 96-well, thin-walled microtiter-style plates (model 6509; Costar Corp., Cambridge, MA) with an Amplitron II thermocycler (Barnstead-Thermolyne, Dubuque, IA). The molecular markers were chosen based on their bin location. The maize RFLP map is divided into "bins" spaced approximately every 20 cM (Gardiner *et al.* 1993). Throughout the paper, we will refer to map location of the markers based on their bin assignments (see UMC 1995 maize RFLP map, Maize DB http://www.agron.missouri.edu/).

Insect bioassay and chemical analysis of selected $F_{2:3}$ families: Forty-five "high apimaysin" and 45 "low apimaysin" families, identified based on chemical analysis of F_2 plants, and six checks (GT114, NC7A, (GT114 $\times$ NC7A)F_1, GT119, Zapalote Chico, and Stowell's Evergreen) were grown in paired-row plots using a randomized complete block design with two replications at Tifton, Georgia during the summer of 1997. Silk masses were collected from plants 3–4 days after emergence of the silks from the husk. Approximately 15 silk masses per family were collected and bulked. $F_{2:3}$ family maysin, apimaysin, and methoxymaysin levels were determined from a sample of the bulked silk masses. The remaining silks were oven dried at 41° for 8 days and used in corn earworm larval bioassays as described in Wiseman (1989) and Byrne *et al.* (1997).

Statistical analysis: Chi-square analysis was used to detect significant ($P < 0.01$) deviation of genotypic classes from the expected 1:2:1 Mendelian segregation ratio. Linkage maps were generated using MAPMAKER/EXP version 3.0 software (Whitehead Institute, Cambridge, MA) for Unix, with a minimum LOD score of 3.0 and a maximum distance of 60 cM. Deviation from normality of the F_2 population for maysin, apimaysin, and total flavone levels was tested using the Shapiro-Wilk statistic (PROC UNIVARIATE, SAS software; SAS Institute, Cary, NC). QTL ($P < 0.001$) affecting maysin, apimaysin, and total flavone (maysin + apimaysin + methoxymaysin) levels were identified using single-factor analysis of variance (ANOVA) (PROC GLM, SAS software; SAS Institute). Genotypic class means were calculated using the least squares means option (LSMEANS) of PROC GLM (SAS software; SAS Institute 1989). Significant ($P < 0.001$) two-way epistatic interactions were identified using EPISTAT (developed by J. B. Holland, Iowa State University, Iowa City, IA). Population size permitted only two-way interactions to be tested. Significant ($P < 0.001$) single loci and two-way interactions were tested in multiple-locus models for maysin, apimaysin, and total flavones. The "best" model was determined to be that which explained the greatest proportion of the phenotypic variance and in which individual loci were significant at $P < 0.001$ and two-way interactions were retained in the model at $P < 0.01$. Significant QTLs were also detected by interval mapping with MAPMAKER/QTL with the threshold value at LOD > 3.0. Simple phenotypic correlation coefficients among traits were computed with the SAS CORR procedure. Within the selected families, loci ($P < 0.001$) affecting 8-day larval weights were identified using single-factor ANOVA. The LSMEANS option was used to calculate genotypic class means for $F_{2:3}$ family 8-day larval weights and for maysin, apimaysin, and total flavone levels.

RESULTS

F_2 population flavone concentration: Frequency distributions showed that essentially all F_2 individuals contained appreciable amounts of maysin, whereas only about one quarter of the individuals contained >0.15% apimaysin (Figure 2), suggesting that apimaysin synthesis may be under recessive, single gene control. Maysin, apimaysin, and total flavone levels were not normally distributed, showing transgressive segregation for high

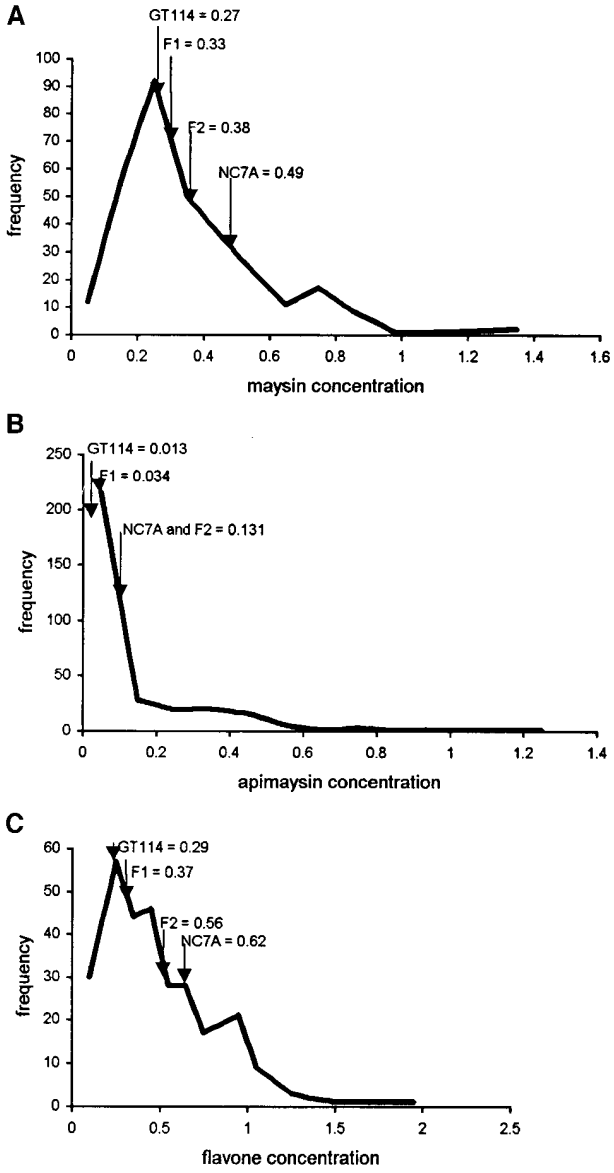

Figure 2.—Frequency distributions of the (GT114 × NC7A) F_2 population for maysin (a), apimaysin (b), and total flavone (c) concentrations in silk tissues. Population, parent, and F_1 means are indicated with arrows.

levels (Figure 2). We did not transform the data to correct for the deviations, thereby maintaining the informativeness of individuals with more extreme values (Mutschler *et al.* 1996).

A framework map of 87 markers covering 1414.2 cM was generated using MAPMAKER/EXP (Figure 3). Segregation ratios were severely distorted ($P < 0.01$) for markers on chromosome *4* (*agrr115* bin 4.01, *umc171a* bin 4.01/4.02, *npi386* bin 4.04, *csu294* bin 4.04/4.05, *umc156* bin 4.06, and *csu907* bin 4.06) in favor of the NC7A allele. Significant segregation distortion ($P < 0.01$) was also observed for three other framework markers: *npi409* (bin 5.01), *asg85* (bin 5.07), and *umc115* (bin 1.01/1.02).

Flavone QTLs: Single-factor ANOVA and MAPMAKER/QTL identified a major QTL on the short arm of chromosome *9* (bin 9.03) affecting maysin levels and a major QTL near the centromere of chromosome *5* (bin 5.05) affecting apimaysin levels (Table 1, Figure 4, a and b). Each of these QTLs was also significant for total flavone levels. By MAPMAKER/QTL analysis, the peak LOD score on chromosome *5* was consistent with the map position of the *pr1* locus. The QTL in the *pr1* region accounted for 64.7 and 26.5% of the phenotypic variation for apimaysin and total flavone levels, respectively. Dominant gene action for low apimaysin was observed for this region, consistent with the expectation that a recessive nonfunctional *pr1* allele is required for apimaysin accumulation. The position of the peak LOD score on chromosome *9* and the gene action, dominant for low maysin, is consistent with the *rem1* locus identified in previous maysin mapping studies (Byrne *et al.* 1996, 1998). By MAPMAKER/QTL analysis, the maysin QTL in the *rem1* region had a peak LOD score of 30.6, accounting for 55.3% of the phenotypic variation in maysin levels and 45.1% of the phenotypic variation in total flavone levels (LOD = 16.9). We conclude that the QTL for apimaysin on chromosome *5* is *pr1* and the QTL for maysin on chromosome *9* is *rem1*. Surprisingly, the QTL for apimaysin on chromosome *5* was not significant for maysin, nor was the maysin QTL on chromosome *9* significant for apimaysin levels (Figure 4, a and b).

We identified three significant ($P < 0.001$) epistatic interactions affecting total flavone levels and three interactions affecting maysin levels that were retained in the multiple-locus models (Table 2). No significant interactions affecting apimaysin were retained in multiple-locus models. The multiple-locus models for maysin and total flavones explained 37 and 41% of the phenotypic variance, respectively (Table 2). Genotypes at linked marker loci were used in the multiple-locus models, resulting in R^2 values lower than the R^2 values associated with the peak LOD score. Only one of the three interactions, *r1* × *umc5*, was retained in the multiple-locus models for both maysin and total flavones. The epistatic interaction between *bnl5.71* (*pr1*) and *wx1* (*rem1*) only affected total flavone levels, even though the two loci involved were the major QTLs for apimaysin and maysin, respectively (Table 1).

Antibiosis QTLs: Significant ($P < 0.001$) correlations were found between $F_{2:3}$ family maysin levels and larval weight ($r = -0.34$) and between $F_{2:3}$ family apimaysin levels and larval weight ($r = 0.48$). The correlation between total flavones and larval weight was not significant. Single-factor ANOVA identified three loci affecting larval weight (Table 3). The *rem1* region on chromosome *9* (*wx1*) and the *pr1* region on chromosome *5* (*bnl5.71*) were significant, accounting for 14.1 and 14.7% of the phenotypic variation, respectively. The locus *umc85* (bin 6.01) on the short arm of chromosome *6* was also sig-

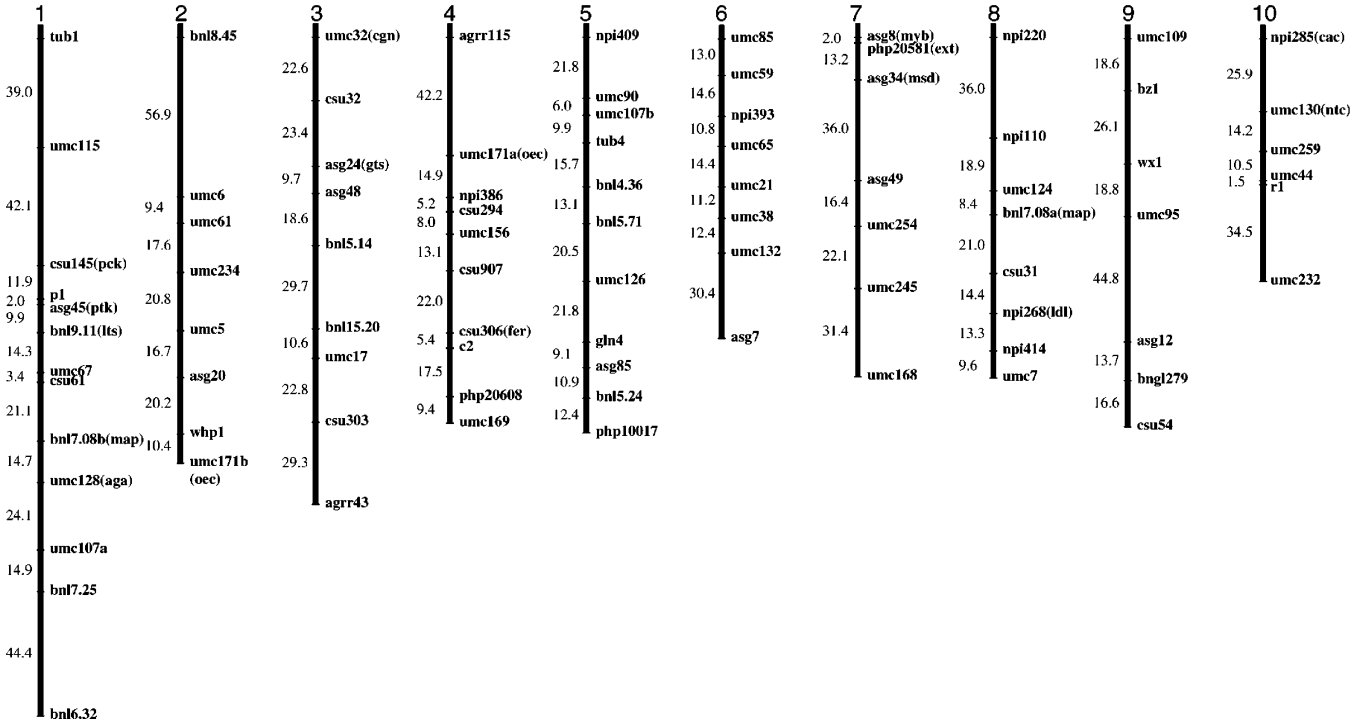

Figure 3.—Framework molecular marker map for (GT114 × NC7A)F_2 population with 87 markers in 10 linkage groups covering 1414.2 cM.

nificant for larval weight, accounting for 15.2% of the phenotypic variation; however, *umc85* was not significant for maysin, apimaysin, and total flavone levels. In the selected $F_{2:3}$ families, the *pr1* region was significant for apimaysin and total flavone levels, and the *rem1* region was significant for maysin, consistent with the F_2 individual results.

DISCUSSION

Single effects and epistasis: What is clear from our results is that the flavone pathway is not nearly as well defined as originally proposed or as simplistic as the anthocyanin pathway has been depicted. Structurally, apimaysin and maysin are highly related compounds, differing only by a 3′-hydroxyl group (apimaysin 3′-H, maysin 3′-OH). Based on the anthocyanin synthesis model, in which all anthocyanins are synthesized from a common pathway, we had assumed that the synthesis of apimaysin and maysin would also occur in a common pathway. We assumed the same structural enzymes, except flavonoid 3′-hydroxylase, and the same pools of metabolic precursors would be required. Instead, the syntheses of apimaysin and maysin appear to be independent. This population was segregating at *pr1*, which is known to affect 3′-hydroxylation of anthocyanins. The genomic region containing *pr1* was detected as the major QTL affecting apimaysin levels. Apimaysin was detected only in individuals homozygous for the nonfunctional NC7A *pr1* allele, the expected consequence of homozygosity for a nonfunctional *pr1* allele. This QTL, however, did not affect maysin levels, which was an unexpected outcome. Apimaysin was not made at the expense of maysin, but rather, silks with apimaysin had increased total flavone levels.

The major QTL for maysin in this population is consistent with the genomic region previously identified as containing the maysin QTL, *rem1*. As in previous studies, a twofold increase in maysin levels was observed in individuals from one of the homozygous *rem1* genotypic classes (Byrne *et al.* 1996, 1998). However, *rem1* did not affect apimaysin levels, again demonstrating an independence of the pathway leading to maysin synthesis from the pathway leading to apimaysin synthesis. The apparent independence of apimaysin synthesis from maysin synthesis suggests that perhaps different precursors are being used or that highly related, yet distinct sets of enzymes are involved.

The two major single effects, *pr1* and *rem1*, are also of interest because of their significant epistatic interaction. Each of the effects alone increased total flavone levels: *rem1* by increasing the amount of maysin, and *pr1* by permitting the synthesis of apimaysin. Individuals homozygous for NC7A alleles at both *rem1* and *pr1* should simultaneously be capable of producing apimaysin and synthesizing additional maysin. However, total flavone levels in the double homozygous class were no higher than with either individual single homozygote effect (Table 1). Even though apimaysin and maysin syntheses appear to be independent of one another, it appears

TABLE 1

Genotype class means for flavone concentrations

			Fresh wt. (%)			LOD R^2 (%)[c]			
Locus	Genotype[a]	Bin	Maysin	Apimaysin	Total[b]	Maysin	Apimaysin	Total	
wx1	A	9.03	0.276[a]	0.124	0.438[a]	30.6 55.3		16.9	45.1
	H		0.311[a]	0.121	0.478[a]				
	B		0.531[b]	0.128	0.715[b]				
bnl5.71	A	5.05	0.395	0.054[a]	0.491[a]		46.0 64.7	15.1	26.5
	H		0.359	0.061[a]	0.459[a]				
	B		0.329	0.300[b]	0.693[b]				

Genotype		Fresh wt. (%)		
bnl5.71	*wxz1*	Maysin	Apimaysin	Total flavone
A	A	0.189[a]	0.029[a]	0.238[a]
A	H	0.329[a]	0.043[a]	0.410[a]
A	B	0.658[b]	0.092[a]	0.817[b]
H	A	0.298[a]	0.063[a]	0.401[a]
H	H	0.319[a]	0.053[a]	0.408[a]
H	B	0.510[b]	0.071[a]	0.632[b]
B	A	0.298[a]	0.346[b]	0.694[b]
B	H	0.280[a]	0.314[bc]	0.671[b]
B	B	0.442[c]	0.240[c]	0.734[b]

Likelihood ration (LOD), chromosomal location, amount of phenotypic variance explained (R^2), and genotypic class least-square means for silk maysin, apimaysin, and total flavones concentrations at *wx1* and *bnl5.71*. Genotypic class least-square means from the *bnl5.71* × *wx1* interaction ($\alpha = 0.001$). Genotypic means with the same letter are not significantly different from one another at $\alpha = 0.001$.

[a] A represents homozygotes for the allele from parent A (GT114), H represents heterozygotes (one GT114 allele and one NC7A allele), and B represents homozygotes for the allele from parent B (NC7A).

[b] Total is the sum of maysin, apimaysin, and methoxymaysin in the silk tissues.

[c] R^2 values are from MAPMAKER/QTL and correspond to the peak LOD score, rather than to the R^2 that is associated with the marker itself.

that an upper limit exists that governs how much total flavone can be produced by the pathway or tolerated by the cell. It should be noted that total flavone levels in some inbred lines are considerably >0.8%. Maysin levels between 1.5 and 2.0% have been observed in some backgrounds (E. Lee, unpublished data), suggesting that the mechanism regulating the ceiling level may be background specific.

B-ring substitution: Maysin is hydroxylated at the 3′-position, and functional *Pr1* is required for 3′-hydroxylation of anthocyanins in maize aleurone tissues (Larson *et al.* 1986). However, allelic constitution differences at *pr1* have no effect on maysin levels. How can maysin be synthesized if *pr1* is not involved? There are several possibilities. First, it is possible that *pr1* is not the QTL, but rather, the apimaysin QTL is tightly linked to *pr1*. It has not been demonstrated that *pr1* encodes flavonoid 3′-hydroxylase, only that anthocyanin synthesis in aleurone tissues requires *pr1* for hydroxylation at the 3′ position (Larson *et al.* 1986). We have worked with other inbred lines that contain functional *p1* alleles in silks and nonfunctional *pr1* alleles. These lines, when grown in the same environment, tend to accumulate a higher proportion of dihydroxy flavones (*i.e.*, maysin) to monohydroxy flavones (*i.e.*, apimaysin) than the nonfunctional *pr1* parent (NC7A) used in this study (NC7A 1.28:1; Tx601 35.3:1; Mp708 3.7:1; PI340853 5.3:1) (E. Lee, unpublished data).

A second possibility is that *pr1* may encode a flavonoid 3′-hydroxylase, but there may be another gene homologous to *pr1* that is also used in maysin synthesis. Maize has many duplicate loci with similar functions, tissue specificities, and/or developmental expression patterns [*e.g.*, *c2* and *whp1* encode chalcone synthase (Franken *et al.* 1991), *r1* and *b1* encode homologous myc-like transcription factors (Chandler *et al.* 1989), and *c1* and *pl1* encode homologous myb-like transcription factors (Cone *et al.* 1993)]. Larson *et al.* (1986) found that sheath tissues of nonfunctional *pr1* plants still retained appreciable amounts of F3′H activity, suggesting the presence of another F3′H. When *pr1* is nonfunctional, maysin is made. Our expectation was that a functional *pr1* would increase maysin. This is not the case. Maysin levels in this population remained unchanged regardless of the constitution at *pr1*.

Finally, it is possible that the B-ring substitution pat-

terns for flavones may occur at the 9-carbon stage rather than the 15-carbon stage. The flavonoid B-ring arises from a common phenylpropanoid pathway intermediate that is generally depicted as being 4-coumaryl-CoA (4-OH). However, chalcone synthase and chalcone isomerase from other species can use caffeoyl-CoA (3,4-diOH) and other 9-carbon substrates in addition to coumaryl-CoA (see Heller and Forkmann 1988; Lui *et al.* 1995). Perhaps caffeoyl-CoA is the precursor used for maysin synthesis, not 4-coumaryl-CoA. If true, this would explain why *pr1* does not behave as a QTL for maysin synthesis and why apimaysin synthesis is independent of maysin synthesis. Two different substrates would be used, caffeoyl-CoA for maysin and 4-coumaryl-CoA for apimaysin. The B-ring hydroxylation would be carried out by caffeic acid-3-hydroxylase (C3H) converting 4-coumarate to caffeic acid. In this scenario, F3′H would function in an alternate pathway that requires 4-coumaroyl-CoA as its substrate. When F3′H is functional, an unknown nonflavone compound is produced. Apimaysin is synthesized only when F3′H is nonfunctional.

Antibiosis: Regardless of allelic constitution at either *rem1* or *pr1*, there was a substantial reduction in larval weight of the experimental group, compared to the larval weight of the control diet group (Table 3). Both the *pr1* and *rem1* regions are QTLs for corn earworm antibiosis. However, the genotypes at *pr1* and *rem1* that result in lower maysin, flavone, and/or apimaysin levels are the genotypes that have lower larval weights (*i.e.*, less flavone, more antibiosis). This is further reflected in the rather poor correlations between the flavones and larval weight. Maysin levels were negatively correlated with larval weight ($r = -0.38$), total flavone levels were not significantly ($P < 0.01$) correlated with larval weights, and apimaysin levels were positively correlated with larval weights ($r = 0.48$).

How can increasing the levels of an antibiotic compound result in apparently less antibiosis? First, total flavone levels of ~0.3% reduce larval growth to near zero. Higher flavone levels show no additional effects because many larvae are already dead. In other populations where *rem1* behaves as a QTL for maysin, it does not behave as an antibiosis QTL, again suggesting that the baseline maysin levels in those populations may be in excess of what is necessary for antibiosis (Byrne *et al.* 1996, 1998). In those populations, however, other maysin QTLs were also QTLs for antibiosis (Byrne *et al.* 1997, 1998). Another possibility is that the additional maysin made through *rem1* and the apimaysin made when *pr1* is recessive comes at the expense of other related antibiotic compounds. The high correlation between maysin levels and larval weights observed by Byrne *et al.* (1996) in a population segregating for a functional *p1* allele indicates that the compounds involved

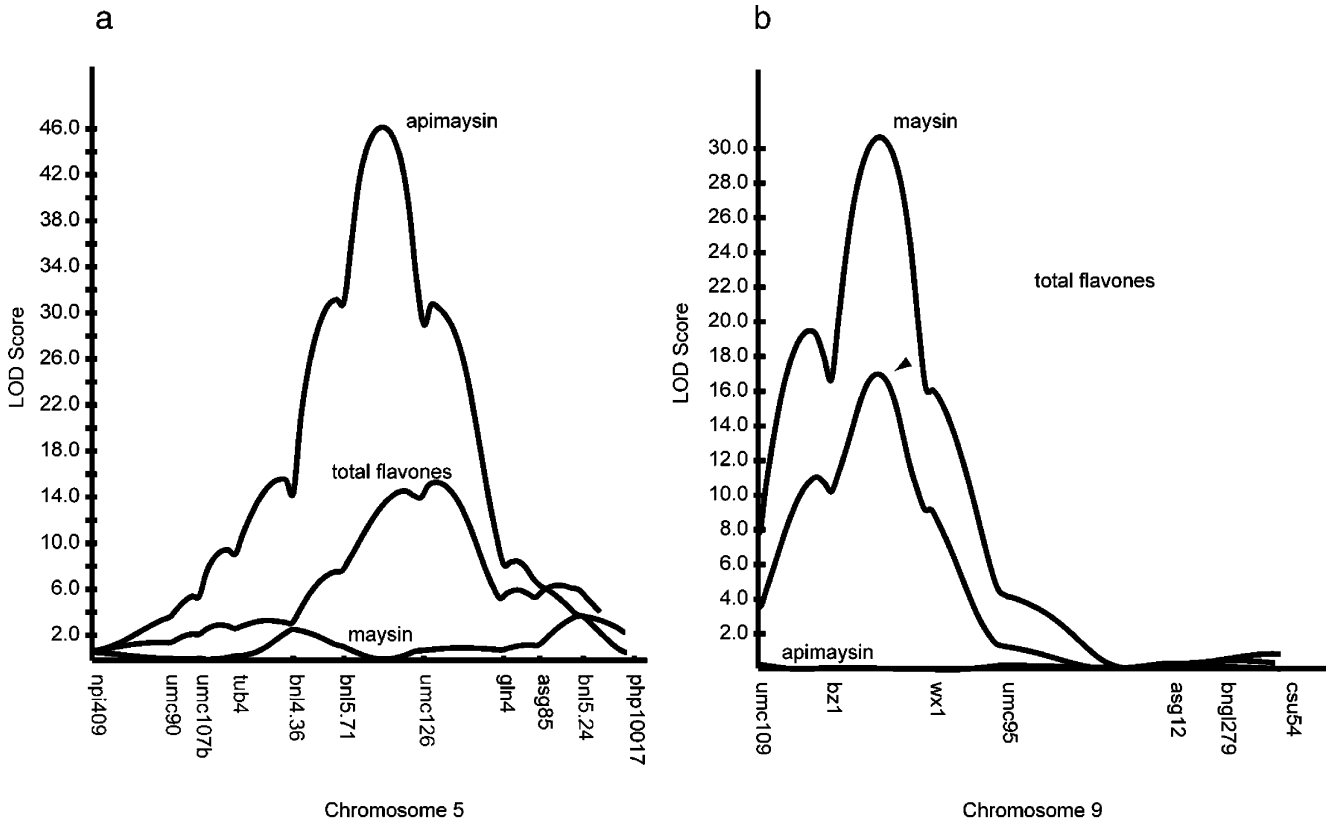

Figure 4.—Composite MAPMAKER/QTL scans of chromosomes *5* (a) and *9* (b) for apimaysin levels, maysin levels, and total flavone levels. Likelihood ratios are on the *y* axis and the framework maps are on the *x* axis.

TABLE 2

Multiple-locus models

Locus or Interaction	Significance ($P<$)
Maysin	
wx1[a]	0.0001
wx1 × *csu31*[b]	0.0001
r1[c] × *umc5*[d]	0.001
npi414[e] × *umc232*[f]	0.01
$R^2 = 0.41$	
$n = 302$	
Total flavones	
wx1	0.0001
bnl5.71[g]	0.0001
bnl5.71 × *wx1*	0.01
asg85[h] × *csu306*[i]	0.01
r1 × *umc5*	0.01
$R^2 = 0.37$	
$n = 300$	

Multiple-locus models for maysin and total flavones with R^2 values and number of observations used in the analyses. Because of missing data points, less than 316 individuals were used in the analyses.

[a] bin 9.03.
[b] bin 8.06.
[c] bin 10.06.
[d] bin 2.07.
[e] bin 8.08.
[f] bin 10.06/10.07.
[g] bin 5.06.
[h] bin 5.07.
[i] bin 4.07/4.08.

in corn earworm antibiosis are under *p1* control. The initial study that identified maysin as an antibiotic factor in silks also found evidence that other nonflavone compounds were involved in corn earworm larval antibiosis (Waiss *et al.* 1979). After the chemical removal of the flavones from silk tissues, Waiss *et al.* (1979) found that compounds extracted in a "hot water" fraction were as effective in larval bioassays as the flavone fraction. The silk residue remaining after removal of both the flavones and the "hot water" fraction retained antibiotic levels equal to the flavone and "hot water" fractions. Other flavonoid-like compounds with a 3′,4′-dihydroxy constitution on the B-ring and a 5,7-dihydroxy constitution on the A-ring have antibiotic activity towards corn earworm larvae, including flavonols, flavanones, and dihydroflavonols (Elliger *et al.* 1980b). These findings suggest that it is not the particular flavonoid class that determines antibiotic levels, but rather the A- and B-ring hydroxylation patterns. One explanation of our larval weight results is that synthesis of additional flavones comes at the expense of one of the other flavonoid compound(s). Furthermore, the positive correlation between apimaysin levels and larval weights may reflect lack of 3′ hydroxylation on the B-ring of flavonols, flavanones, and dihydroflavonols, rendering them less effective against corn earworm larvae.

Conclusions: Although the genetic basis of the variation in synthesis of maysin and apimaysin for this population was superficially simple, one major QTL explaining the majority of the variation for each chemical, this study revealed a number of important points about flavonoid synthesis and the biological interpretations of QTL analyses. First, the model for anthocyanin/flavonol synthesis does not necessarily fit C-glycosyl flavone synthesis. The anthocyanin/flavonol synthesis model depicts B-ring substitutions occurring at the 15-carbon stage, as well as the sharing of substrates and enzymes between pathways. For C-glycosyl flavone synthesis, this is not the case. Second, synthesis of highly related compounds

TABLE 3

Genotype class means for larval weights and flavones

Locus	Genotype	Bin	Larval wt. (mg)	R^2 (%)	Fresh wt. (%)		
					Maysin	Apimaysin	Total flavones
wx1	A	9.03	97.61[a]	14.1	0.170[a]	0.200	0.370
	B		170.62[b]		0.291[b]	0.164	0.456
	H		91.99[a]		0.187[a]	0.199	0.387
bnl5.71	A	5.05	76.28[a]	14.7	0.243	0.046[a]	0.289[a]
	B		146.89[b]		0.183	0.313[b]	0.497[b]
	H		85.03[a]		0.172	0.167[c]	0.340[a]
umc85	A	6.01	161.49[a]	15.2	0.178	0.214	0.392
	B		79.34[b]		0.212	0.143	0.356
	H		91.78[b]		0.210	0.206	0.416
Control diet			743.50				

Chromosomal location, amount of phenotypic variance explained (R^2), and genotypic class means of loci significantly associated with 8-day larval weights and the corresponding means for maysin, apimaysin, and total flavone concentrations from the selected $F_{2:3}$ families. Genotypic means with the same letter are not significantly different from one another at $\alpha = 0.01$.

within a pathway, and presumably similar effects on traits, can appear to have independent genetic control. Therefore, identification of different QTLs in separate populations cannot be interpreted to mean that different genetic systems or pathways affect trait expression.

The third point reinforced in this study is the importance of considering related pathways in explaining QTL effects. Is the increase in maysin through *rem1* and the synthesis of apimaysin through *pr1* coming at the expense of other antibiotic compounds? The genetic mechanism underlying the increase in maysin by *rem1* is unknown, and, at best, the role of *pr1* in maysin synthesis is not entirely clear. However, the lack of correlation between the increased flavone levels through *rem1* and *pr1* and larval weight suppression suggests that either the additional flavones are made at the expense of other antibiotic compounds, or that the population's baseline flavone level is sufficient to cause larval death. Because of the very high correlation between maysin levels and larval weight when variation for maysin levels results from segregation of a functional *vs.* nonfunctional *p1* allele (Byrne *et al.* 1996, 1997), we suspect that if there are "unknown antibiotic compound(s)" involved, that they are also under *p1* control.

Finally, epistatic interactions may represent at least two distinct mechanisms: first is the more commonly considered complementary gene action affecting a single process (trait), and second is the specification of conflicting processes that cannot be simultaneously accomplished by cellular metabolic systems. A surprising result of this study was the nature of the epistatic interaction involving the two single-effect QTLs, *rem1* and *pr1*. The interaction was only significant for "total flavones," not for the individual chemicals themselves. This interaction indicates the presence of a mechanism governing total flavone levels. The synthesis of maysin and apimaysin was independent only as long as total flavone synthesis is under this ceiling level. The phenylpropanoid pathway, flavone synthesis, and corn earworm antibiosis continue to serve as excellent models for investigating and interpreting quantitative genetic theory in relation to a known biological system, demonstrating the interconnected dynamic nature of the pathway and the phenotypic consequences.

Note added in proof: Chemical analysis of silks from the test cross of NC7A × *pr1* tester revealed that recessive *pr1* is not sufficient for apimaysin synthesis.

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